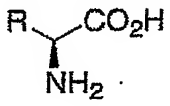
Process for preparing optically active amino acids using a whole-cell catalyst

The invention describes a process for preparing optically active L-α-amino acids. In particular the present invention describes a process for preparing compounds of the general formula (I)



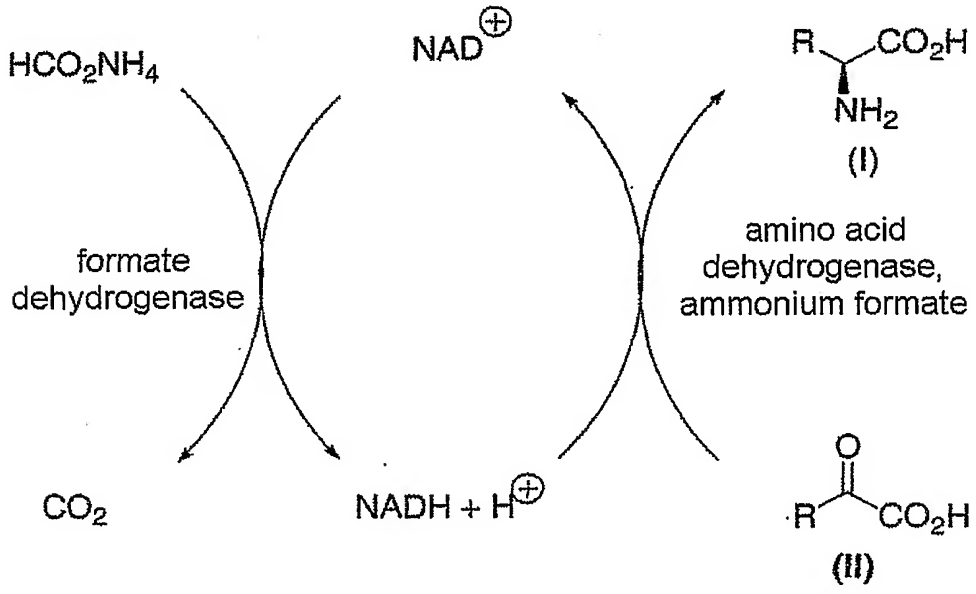
(I),

in which R is alkyl, in particular a space-filling branched alkyl group which exhibits a tertiary C atom and which possesses 5-10 C atoms, for example tert-butyl, and substituted alkyl, or salts which are derived therefrom.

Optically active L-α-amino acids are used for preparing a number of valuable compounds. For example, these compounds function as intermediates in the production of pharmaceuticals. L-tert-Leucine, which can be found as a structural element in a number of pharmaceutical active compounds and is consequently required as an intermediate for synthesizing the corresponding pharmaceutical active compounds, is a particularly valuable representative of this product class. A. S. Bommarius et al., (J. Mol. Cat. B: Enzymatic 1998, 5, 1-11) provides examples of uses of L-tert-leucine as a building block for pharmaceutical active compounds.

Using a leucine dehydrogenase and a formate dehydrogenase from Candida boidinii to enzymically reduce 2-ketocarboxylic acids while regenerating cofactor in situ constitutes an industrially

established method for preparing optically active L-α-amino acids. In particular, this route is suitable for preparing the non-proteinogenic amino acid L-tert-leucine, which is produced on the ton scale using this biocatalytic method. The method is described in detail in the literature (EP0692538; U. Kragl, D. Vasic-Racki, C. Wandrey, Bioprocess Engineering 1996, 14, 291-297; A. S. Bommarius, M. Schwarm, K. Drauz, J. Mol. Cat. B: Enzymatic 1998, 5, 1-11; G. Krix, A. S.: Bommarius, K. Kottenhahn, M. Schwarm, M.-R. Kula, J. Biotechnol. 1997, 53, 29-39, A. Liese, C. Wandrey, A. Liese, K. Seelbach, C. Wandrey, Industrial Biotransformations, Wiley-VCH, Weinheim, 2000, p. 125f. and A. S. Bommarius, K. Drauz, W. Hummel, M. -R. Kula, C. Wandrey, Biocatalysis 1994, 10, 37-47. In addition, a general review is provided in A. S. Bommarius in: Enzyme Catalysis in Organic Synthesis (Eds.: K. Drauz and H. Waldmann), Volume 2, 2nd edition, Wiley-VCH, Weinheim, 2003, chapter 15.3, p. 1047f.).



Scheme 1. Preparation of L-tert-leucine using isolated enzymes and added cofactor (taking as an example an NAD^+-dependent amino acid dehydrogenase and a formate

dehydrogenase for regenerating cofactor)

Typical quantities of NAD+ cofactor which are used, and which have to be added, are described, for example, in EP0692538 and are in the range of from 0.0008 equivalents to 0.02 equivalents. In addition, G. Krix et al. (J. Biotechnol. 1997, 53, 29-39) describe the preparation of (S)-neopentylglycine in industrial batch sizes using an NAD^+ cofactor quantity of 0.003 equivalents. Typical substrate concentrations in EP0692538 are 100-250 mM. A. Liese et al. (Industrial Biotransformations, Wiley-VCH, Weinheim, 2000, p. 125f.) describe the preparation of L-tert-leucine using a substrate concentration of 0.5 M and with a yield of 74%. G. Krix et al. (J. Biotechnol. 1997, 53, 29-39) also describe the performance of reductive aminations using isolated leucine dehydrogenase and formate dehydrogenase enzymes at substrate concentrations of from 0.5 to 1 M.

The high turnovers and outstanding enantioselectivities, which are > 99% ee and consequently help to meet the strict quality demands placed on pharmaceutical intermediates, are advantageous features of this method. It is also possible to operate at high substrate concentrations, something which is an important aspect particularly from the industrial point of view.

However, a disadvantage of the previous method is, in the first place, the requirement for isolated enzymes. These latter are used, in particular, in purified form, with this being accompanied by an increase in the share of the costs due to the biocatalyst. Because of the high enzyme costs resulting from this, it is necessary to recycle the enzymes many times in order to obtain a favorable process economy, in particular low enzyme costs. In addition to the long running times of these

recycling procedures, which are advantageously carried out continuously, the relatively small reaction volumes per batch which result from this are disadvantageous.

Another disadvantage is the requirement for cofactor which is added in the reaction. While these cofactors are added catalytically in orders of size of approx. 0.001 equivalents, they nevertheless represent, because of their high price, a considerable cost factor even at catalytic quantities.

A process in which the necessity of using isolated enzymes and of adding cofactor is dispensed with, or the addition of cofactor is kept to a minimum, and the synthesis nevertheless proceeds with a high turnover rate, high enantioselectivity and high volumetric productivity, would therefore be desirable. In this way, it would be possible to lower enzyme costs considerably and save on cofactor costs, and consequently increase the economy of the process.

Soda et al. describe the use of a whole-cell catalyst, comprising a leucine dehydrogenase and a bacterial formate dehydrogenase, in the reductive amination of, inter alia, branched-chain α-ketocarboxylic acids such as L-tert-leucine (Appl. Environm. Microbiology 1997, 63, 4651-4656). This publication explicitly points out that the enzymes which are required in the reductive amination can be used in the form of a whole-cell catalyst, in particular E. coli, as live or resting cells, which comprises these enzymes. However, if preference were to be given to taking advantage of the intracellular pool of NAD^+ in E.coli, for the purpose of avoiding having to add the NAD^+, the final concentration of product would then be restricted to about 0.3 M. This is not adequate for industrial applications.

The object of the present invention was therefore to specify another process for preparing L-α-amino acids which operates enzymically and which can be carried out advantageously on an industrial scale. The process should, in particular, be superior to the processes of the prior art with the abovedescribed aspects and should make it possible to produce the desired products advantageously from the point of view of process economics (in particular space-time yield).

These objects, and other objects which are not specified in more detail but which ensue from the prior art in an obvious manner, are achieved by a process having the features of the present claim 1. Claims 2 to 9 are directed preferred embodiments of the present process.

Said object is achieved, in a manner which is extremely elegant and surprising but nonetheless advantageous for that, by, in a process for preparing enantiomerically enriched L-α-amino acids or their salts by reacting the corresponding 2-ketocarboxylic acid with an ammonium ion donor in the presence of a whole-cell catalyst which comprises a cloned gene encoding a cofactor-dependent amino acid dehydrogenase and a cloned gene encoding an enzyme which regenerates the cofactor, metering, at a total input of substrate per reaction volume of ≥ 500 mM, the addition of the substrate such that the stationary concentration of 2-ketocarboxylic acid is less than 500 mM and the external addition of cofactor, based on the total input of substrate, corresponds to < 0.0001 equivalents.
Surprisingly, it is possible, for example by using the whole-cell catalyst while at the same time metering in the substrate, to dispense with any addition of the expensive cofactor or, by means of making a minimal external addition (< 0.0001 equivalents), to keep its concentration in a low range, with this helping to save

on process input costs. By contrast, without this metering technology and when initially introducing substrate quantities per reaction volumes of > 500 mM, the reductive amination using the whole-cell catalyst only succeeds when relatively large quantities of the NAD+ cofactor are added. In the absence of the latter, the concentration only proceeds unsatisfactorily (see comparative example "synthesis example 1", initial substrate quantity per reaction volumes 900 mM - final turnover 25%). It is consequently only by using the process according to the invention (see synthesis examples 2 to 5) that it is possible to be able to almost completely dispense with the external addition of the cofactor even when carrying out the synthesis with relatively high total turnover quantities per reaction volumes and consequently under conditions which make sense from the point of process economics.

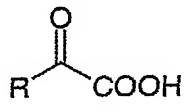
In a preferred embodiment, the expensive cofactor is therefore only added in quantities which are such that a concentration of preferably < 0.00005 equivalents, extremely preferably < 0.00001 equivalents, based on the substrate, is maintained. Very particular preference is given to an embodiment in which no cofactor is added externally to the reaction mixture. In this case, therefore, no addition of the cofactors (e.g. NAD(H)) need take place at all, something which it was not possible to deduce in an obvious manner from the prior art.

Within the context of the reaction under consideration, the skilled person is free to choose the genes which encode a cofactor-dependent amino acid dehydrogenase and an enzyme which regenerates the cofactor, which genes are to be expressed by the whole-cell catalyst, as host organism. He will lean toward enzymes which are known from the prior art.

With regard to the amino acid dehydrogenase, suitable enzymes are, in particular, those which are selected from the group consisting of leucine dehydrogenases (like in US5854035) and phenylalanine dehydrogenases (like in US5416019). Amino acid dehydrogenases (the latter e. g. in A. Bommarius in: Enzyme Catalysis in Organic Synthesis (Eds.: K. Drauz and H. Waldmann), Volume III, Wiley-VCH, Weinheim, 2002, chapter 15.3) which have proved to be suitable are, in particular, the leucine dehydrogenases, with the leucine dehydrogenases from Bacillus strains, and, in this case, in particular, from *Bacillus sphaericus*, *Bacillus cereus* (Seq. ID No. 5) and *Bacillus stearothermophilus* being particularly suitable. Cofactor-regenerating enzymes which can be taken into consideration are those selected from the group consisting of formate dehydrogenases (like in EP1295937), malate dehydrogenases (like in PCT/EP/03/08631), lactate dehydrogenases and glucose dehydrogenases (the latter, by way of example, in A. Bommarius in: Enzyme Catalysis in Organic Synthesis (eds.: K. Drauz and H. Waldmann), Volume III, Wiley-VCH, Weinheim, 2002, p. 1473, 993, 994, 1037, 1038, 1054, 1126; Glucose dehydrogenase from Bacillus subtilis expressed in Escherichia coli. I: Purification, characterization and comparison with glucose dehydrogenase from Bacillus megaterium, Hilt W; Pfleiderer G; Fortnagel P Biochimica and biophysica acta (1991 Jan 29), 1076(2), 298-304). The use of a formate dehydrogenase from *Candida boidinii* or mutants resulting therefrom (like in EP1295937; Seq. ID No. 7), while employing a formate-containing component as substrate, has proved to be very particularly preferred.

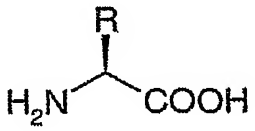
In this connection, a whole-cell catalyst which comprises a leucine dehydrogenase and a formate dehydrogenase from *Candida boidinii* or mutants derived therefrom is particularly suitable.

The substrate spectrum which is converted by the whole-cell catalyst differs depending on the amino acid dehydrogenase which is employed. While the leucine dehydrogenase is more suitable for linear and branched aliphatically substituted 2-ketocarboxylic acids, the phenylalanine dehydrogenase is preferably used for aromatic substituted substrates. With regard to the use of leucine dehydrogenase in the whole-cell catalyst, it is preferably possible to employ and convert substrates of the general formula (II) possessing an aliphatic radical R



(II)

Substrates which possess bulky aliphatic radicals as R are particularly suitable. These R radicals are primarily those selected from the group consisting of 1-adamantyl, neopentyl and tert-butyl. For this reason, preference is given to a process in which use is made of 2-ketocarboxylic acids, or salts resulting therefrom, which yield amino acids of the general formula (I)



(I)

in which R is alkyl, in particular a space-filling branched alkyl group which exhibits a tertiary C atom and possesses 5-10 C atoms, for example tert-butyl, and substituted alkyls.

In principle, the skilled person is free to choose the manner in which he carries out the process according to the invention. In this connection, he will lean toward processes which are known from the prior art. These processes can be continuous or discontinuous. It is advantageous to meter the addition of the substrate in accordance with a fed batch process [see, for example, synthesis examples 2 and 4] or by continuously adding it [see, for example, synthesis example 3 and 5, respectively]. In both process variants, the substrate is added such that the stationary concentration of substrate is less than 500 mM.

It has turned out to be advantageous to use the 2-keto-carboxylic acid employed as substrate at a maximum stationary concentration of less than 450 mM, and very particularly preferably of less than 400 mM, during the reaction.

In the fed batch process, the substrate is added in portions, after given units of time and preferably as a substrate solution. The number of the substrate portions which are added is preferably between 3 and 15, very preferably between 5 and 9. The concentration of the added substrate solution should preferably be set high enough to achieve a total input of substrate per reaction volume which is as high as possible. Synthesis examples 2 and 4 provide examples of this fed batch process variant. In the case of the continuous process variant, the substrate is added continuously over a given period of time, preferably at a constant metering rate, with the substrate preferably being added in the form of a substrate solution. Synthesis example 3 provides an example of this continuous process variant.

All known cells are suitable for use as the whole-cell catalyst which comprises an amino acid dehydrogenase

and an enzyme which is capable of regenerating the cofactor. Microorganisms which may be mentioned in this regard are organisms such as yeasts, such as *Hansenula polymorpha*, *Pichia sp.*, *Saccharomyces cerevisiae*, prokaryotes, such as *E.coli* and *Bacillus subtilis*, or eukaryotes, such as mammalian cells, insect cells or plant cells. The methods for cloning are well-known to the skilled person (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York). Preference is given to using *E.coli* strains for this purpose. Those which are very particularly preferred are: E.coli XL1 Blue, NM 522, JM101, JM109, JM105, RR1, DH5α, TOP 10-, HB101, BL21 codon plus, BL21 (DE3) codon plus, BL21, BL21 (DE3), MM294. Plasmids which can preferably be used to clone the gene construct containing the nucleic acid according to the invention into the host organism are likewise known to the skilled person (see also PCT/EP03/07148; see below).

Suitable plasmids or vectors are, in principle, all the versions which are available to the skilled person for this purpose. These plasmids and vectors can be found, for example, in Studier and coworkers (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendroff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89) or the brochures provided by the companies Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Other preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: a practical approach, Vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R. L. and Denhardt, D. T. (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular

cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York. Plasmids which can very preferably be used to clone the gene constructs containing the nucleic acid sequences under consideration into the host organism are, or are based on: pUC18/19 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene or pET (Novagen).

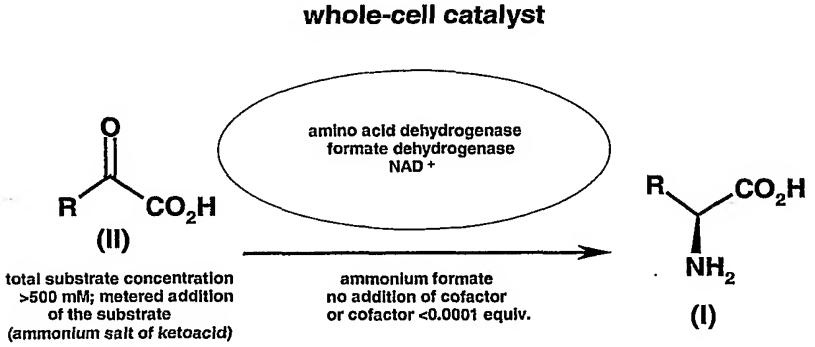
In another embodiment of the process according to the invention, before it is used, the whole-cell catalyst is preferably pretreated such that the permeability of the cell membrane for the substrates and products is increased as compared with the intact system. In this connection, particular preference is given to a process in which the whole-cell catalyst is, for example, pretreated by being frozen and/or by being treated with toluene. The essential features of the process according to the invention are shown in scheme 2.

The substrates can be employed at an extraordinarily high concentration when using the present process, as has also been described in the prior art when using the individual enzymes. In the present case, it is advantageous to employ the 2-ketocarboxylic acid at a concentration of greater than 500 mM. It is also preferred to introduce the substrate into the reaction at concentrations of greater than 800 mM, preferably greater than 900 mM and very particularly preferably greater than 1000 mM. However, in the case of this embodiment, it is essential to add cofactor to the reaction mixture in order to achieve corresponding turnover rates.

If, however, it is wished, despite a high space-time yield being demanded, to use the whole-cell catalyst such that it does not become necessary to add the expensive cofactor externally, or only necessary to

make an extremely small external addition of less than 0.0001 equivalents, the skilled person can then surprisingly achieve this by the metering, in accordance with the invention, of the substrate.

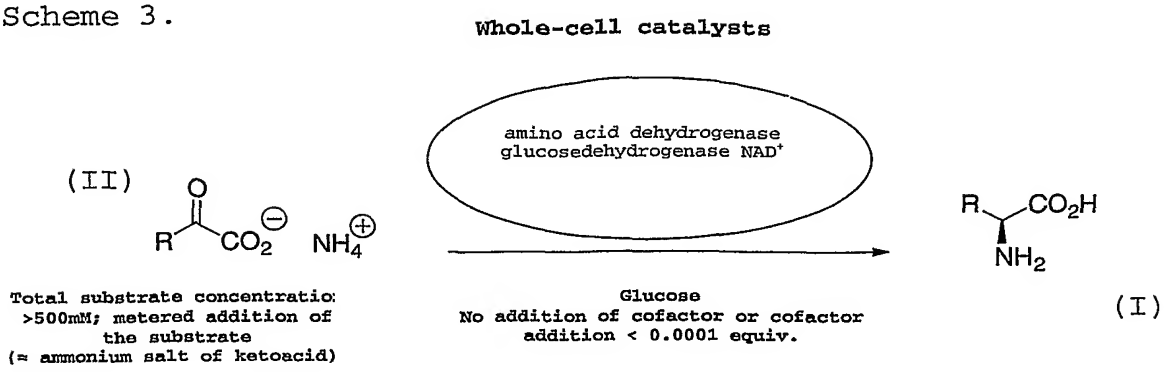
In the case of the present reaction, the procedure is preferably that the whole-cell catalyst and the ammonium ion donor are initially introduced in water. Any compound which is suitable to the skilled person for this purpose can be used as the ammonium ion donor. In particular, these ammonium ion donors are compounds which are selected from the group consisting of typical ammonium salts. Very particular preference is given to using ammonium formate when a formate dehydrogenase is selected as the cofactor regeneration system or the ammonium salt of the respective ketoacid. The reaction can be depicted very clearly by means of the following scheme 2.



Scheme 2. Principle of the reaction in the whole-cell catalyst process according to the invention (taking as an example an NAD^+-dependent amino acid dehydrogenase and a formate dehydrogenase for regenerating cofactor)

In a further preferred embodiment the whole-cell catalyst embracing a glucose dehydrogenase and an amino acid dehydrogenase is mixed with water and glucose and the ammonium salt of the respective ketoacid is

subjected thereto. The reaction is shown in subsequent Scheme 3.



(I)

Scheme 3. Reaction of whole-cell catalyst of the invention, e. g. by way of an NAD^+-dependent amino acid dehydrogenase and a glucose dehydrogenase for regeneration of the cofactor.

If other dehydrogenases are used instead of the leucine dehydrogenase, the conditions under which the enzyme in question functions optimally can be found in the prior art. The reader is referred to US5416019 and Galkin et al. (Appl. Environ. Microbiol. 1997, 63, 4651) with regard to using a phenylalanine dehydrogenase.

With regard to the cofactor-regenerating enzymes and the conditions to be established, reference can be made to EP1295937 (formate dehydrogenase), PCT/EP/03/08631 (malate dehydrogenase) and Enzyme Catalysis in Organic Synthesis (Eds.: K. Drauz and H. Waldmann), Volume III, Wiley-VCH, Weinheim, 2002, S. 1473, 993, 994, 1037, 1038, 1054 or 1126. Further glucose dehydrogenase from Bacillus subtilis expressed in E. coli is preferred (I: Purification, characterization and comparison with glucose dehydrogenase from Bacillus megaterium, Hilt W; Pfleiderer G; Fortnagel P, Biochimica et biophysica acta (1991 Jan 29), 1076(2), 298-304) and literature cited therein.

The reaction mixture is worked up using methods known to the skilled person. In the batch process, the biomass can be readily separated from the product by means of filtration or centrifugation. The amino acid which is obtained can then be isolated using customary methods (ion exchange chromatography, crystallization).

However, the present process can also be carried out continuously. For this, the reaction is carried out in what is termed an enzyme-membrane reactor in which high molecular weight substances, i.e. the biomass, are retained behind an ultrafiltration membrane and low molecular weight substances, such as amino acids which have been produced, are able to pass through the membrane. A procedure of this nature has already been described several times in the prior art (Wandrey et al. in year-book 1998, Verfahrenstechnik und Chemieingenieurwesen [Process technology and chemical engineering], VDI, p. 151ff; Kragl et al., Angew. Chem. 1996, 6, 684).

The process, which is presented here, for preparing amino acids, which are, in particular, bulky, can very readily be established on a commercial scale on account of its advantages. The surprising fact that the addition, which is necessary in the case of the reaction under consideration, of a cofactor can be dispensed with in the process according to the invention, as well as the advantages arising from the fact that the whole-cell catalysts are easy to manage, constitute the non-obvious superiority of the present invention over the methods of the prior art.

Furthermore, it can be regarded as being surprising that the influence of undesirable metabolic/physiological functions is of no importance when using the whole-cell catalyst. Both aspects help, in an extraordinarily comprehensive manner, to lower the

process costs entailed in preparing the L-α-amino acids.

It is furthermore surprising that, despite permeabilization of the cell wall and the possibility, associated therewith, of the cofactor present in the cells escaping, a negative impairment of the reaction which might be expected, for example as a result of the turnover being decreased, is not observed.

Within the context of the invention, optically enriched (enantiomerically enriched, enantiomer enriched, enantiomerically pure) compounds are understood as meaning the presence of one optical antipode at > 50 mol% when mixed with the other.

The whole-cell catalyst is understood as meaning a microorganism which comprises cloned genes which encode enzymes which are at least able to catalyze two consecutive steps in the transformation of an organochemical compound. In this regard, and with regard to the general preparation methods (matching the enzyme expression with regard to the turnover rates), the reader is referred to EP1216304.

According to the invention, alkyl is understood as meaning a (C_1-C_{18})-alkyl radical. This encompasses linear and arbitrarily branched radicals of this nature. It includes, in particular, methyl, ethyl, 1-propyl, 2-propyl, 1-n-butyl, 2-n-butyl, 1- or 2-isobutyl, 1- or 2-sec-butyl, tert-butyl, etc. The radicals can be substituted once or more than once by (C_1-C_8)-heteroalkyl radicals or radicals such as OH, SH, Hal and NH_2. Heteroalkyl radicals are understood as meaning, in particular, an alkyl radical as described above which possesses from 1 to 8 C atoms and which contains heteroatoms, such as O, S or N in its chain or

which is bonded, by way of these heteroatoms, to the molecule under consideration.

External addition of cofactor means that this quantity of cofactor is added artificially to the reaction mixture. This quantity is to be seen as being in addition to the quantity of cofactor which is already inherently introduced into the reaction mixture by the whole-cell catalyst.

It goes without saying that the 2-ketocarboxylic acid which is used in the reaction is present in the reaction mixture in dissociated form. This form can be obtained either by using the ketocarboxylic acid and adjusting the pH correspondingly or by adding the salts of the ketocarboxylic acids. Both forms are included here analogously and in accordance with the invention.

The term total substrate concentration stands for the total input of substrate per reaction volume.

Figures:

Fig. 1 - pAM3.25 (Seq. ID No. 9):

Construction of pJOE4580.2

The plasmid pJOE4580.2 was formed from the published plasmid pJOE3075 (T. Stumpp, B. Wilms and J. Altenbuchner (2000) Biospektrum 1/2000: 33-36) by removing the malE gene by cutting with the restriction endonucleases NdeI/HindIII and replacing it with two oligonucleotides which once again complemented the NdeI and HindIII cleavage sites and, in addition to this, carried an NheI, an AatII and a PstI cleavage site. A SmaI fragment from the plasmid pJOE773 (J. Altenbuchner, P. Viell, I. Pelletier (1992) Positive selection vectors based on palindromic DNA sequences. Methods Enzymol 216: 457-466), which fragment carries the E.coli lacZalpha gene, was inserted into the NheI cleavage site after filling using Klenow polymerase and dNTPs. When harboring this plasmid, E. coli JM109 gives blue colonies on LB plates containing X-Gal and IPTG. This plasmid was named pJOE4580.2. The FDH sequence (Seq. ID No. 7) was cloned into this plasmid. The resulting plasmid was named pAM3.25.

Fig. 2 - pAM5.22

Construction of pJOE4580.2

The plasmid pJOE4580.2 was formed from the published plasmid pJOE3075 (T. Stumpp, B. Wilms and J. Altenbuchner (2000) Biospektrum 1/2000: 33-36) by removing the malE gene by cutting with the restriction endonucleases NdeI/HindIII and replacing it with two oligonucleotides which once again complemented the NdeI and HindIII cleavage sites and, in addition to this,

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Fig. 3 - pAM8.21

Construction of pHWG640.12 (Seq. ID No. 11)

Plasmid pHWG640.12 has not previously been published and its construction is therefore described as follows. This plasmid pHWG640.12 is constructed proceeding from the published plasmid pAW229 in a manner which is readily reworkable. Plasmid pAW229 is a pACYC184 derivative which contains a rhamnose promoter. Proceeding from pAW229 (B. Wilms, A. Wiese, C. Syldatk, R. Mattes, J. Altenbuchner (2001) J. Biotechnol 86: 19-30), the hyuC gene was excised from the plasmid with NdeI/HindIII and replaced with a PCR fragment which was cut with the same restriction enzymes and which contains the E. coli K12 sfcA (malic enzyme) gene. The resulting plasmid was designated pHWG640.12. The LeuDH sequence was inserted into this plasmid. The new plasmid is named pAM8.21.

Fig. 4 - pAM10.1 (Seq. ID No. 10)

The scfA gene (Seq. ID No. 11) was deleted from plasmid pAM8.21. The new plasmid is named pAM10.1.

Fig. 5
Biocatalyst with depiction of the course of the specific activity of leucine dehydrogenase (LeuDH) and formate dehydrogenase (FDH), and of the optical density, in dependence on the induction time; for a detailed description of the fermentation conditions, see experimental section.

Experimental examples

Preparing the whole-cell catalyst

Gene amplification and cloning

In order to clone the formate dehydrogenase (FDH, fdh3 from *Candida boidinii*, mutant with lower sensitivity to oxidation) and leucine dehydrogenase (*Bacillus cereus* LeuDH) for the whole-cell catalysis of the conversion of trimethylpyruvate into tert-leucine with regeneration of cofactor, the genes for the two enzymes were first of all amplified by PCR from chromosomal DNA obtained from the abovementioned strains. The oligonucleotides employed are listed in Table 1 while the composition of the PCR mixtures is given in Table 2 and the PCR program is given in Table 3.

Table 1: Oligonucleotides for amplifying the FDH and LeuDH genes

Oligo-nucleotide	5'-3' sequence		Seq. ID No.
s3713	AAA AAA CTT AAG AAG GAG ATA TAC ATA TGA CAT TAG AAA TCT TCG AA	LeuDH forward	1
s3714	AAA AAA CTG CAG TTA GCG ACG GCT AAT AAT AT	LeuDH reverse	2
s3723	AAA AAA CAT ATG AAG ATT GTC TTA GTT CTT	FDH forward	3
s3716	AAA AAA GAC GTC TTA TTT CTT ATC GTG TTT ACC	FDH reverse	4

The oligonucleotides were used to append cleavage sites for restriction endonucleases to the genes. These are BfrI in the case of s3713, PstI in the case of s3714, NdeI in the case of s3723 and AatII in the case of s3716 (see underlined regions).

Table 2: PCR mixtures, polymerase, buffer and $MgCl_2$ originate from the company Biomaster; the plasmid DNA starting concentration was 50 µg/ml

Component	For FDH	Mixture for FDH	for LeuDH	Mixture for LeuDH
Plasmid DNA from strain FDH-C235/C262A		2 µl	pLeu2 plasmid DNA	2 µl
10× buffer		10 µl		10 µl
50 mM MgCl2		3 µl		3 µl
100% DMSO		10 µl		10 µl
10 mM dNTPs		2 µl		2 µl
33 mM oligo 1	S3723	1 µl	s3713	1 µl
33 mM oligo 2	s3716	1 µl	s3714	1 µl
Taq polymerase		1 µl		1 µl
Deionized H2O		70 µl		70 µl

Table 3: PCR program: steps 2 to 4 were repeated 30 times

Step	T, t for FDH amplification	T, t for LeuDH amplification
1. Denaturation of the DNA	94°C, 5 min	94°C, 5 min
2. Oligo annealing	50°C, 1 min	51°C, 1 min
3. DNA elongation	72°C, 1: 30 min	72°C, 1:30 min
4. Denaturation of the dsDNA	92°C, 1 min	92°C, 1 min
5. DNA elongation	72°C, 7 min	72°C, 7 min

After the gene amplification, the PCR fragments were purified using the "DNA PCR and gel band purification kit" supplied by the company GFX and ligated into the L-rhamnose-inducible vectors pJOE4580.2 (pBR322 derivative; Fig. 1) and, respectively, pHWG640.12 (pACYC184 derivative; Fig. 3; Seq. ID No. 11) using the restriction endonucleases mentioned below.

In general, restriction mixtures were prepared using approx. 50 µg of DNA/ml in the 10 µl standard mixture. 1 µl of the first enzyme, and 1 µl of the 10× concentrated enzyme buffer, were also added. The mixtures were adjusted to the final volume using deionized H2O. The DNA to be inserted was incubated with the restriction enzymes separately from the plasmid DNA. After the restriction with the first enzyme, there then followed a precipitation step in which the DNA was precipitated with isopropanol and washed with ethanol and then dried and taken up in 8 µl of TE 10.01. In each case 1 µl of the second enzyme and 1 µl of the second 10× enzyme buffer were added to these mixtures, which were incubated once again at 37°C for 1.5 h. The vector pAM10.1 was prepared from pAM8.21, this was also followed by a treatment with

Klenow polymerase. The DNA was then separated into its fragments using a 1% agarose gel (Seakem agarose containing 0.4 µg of ethidium bromide/ml) and the correct bands were excised with a scalpel for further use. The DNA was eluted, in accordance with the instructions, from the small gel blocks using the "EASY PURE gel purification kit" supplied by the company Biozym and taken up in 15 µl TE 10.01.

For the ligation of vector and insert, the mixtures were selected such that the insert DNA was present at approximately twice the concentration of the target vector. In this case, too, the DNA-concentration was approx. 50 µg/ml. The final volume of the ligation mixtures was 10 µl, with the mixtures also containing 1 µl of ligase and 1 µl of 10× concentrated ligase buffer (both from ROCHE) in addition to the vector/insert mixture. The incubation took place overnight at 4°C. The ligation mixtures were transformed into E.coli K12 JM109, with this bacterium then being selected on LB agar containing antibiotics (100 µg of ampicillin/ml (pAM3.25 [Seq. ID No. 9], pAM5.22) or 25 µg chloramphenicol/ml (pAM8.21, pAM10.1 [Seq. ID No. 10]), and clones were checked for the expected plasmid after the plasmids had been isolated.

Since LeuDH (Seq. ID No. 6) was initially to be coupled to malic enzyme (Seq. ID No. 12), the LeuDH gene was first of all inserted into pJOE4625.1, which already contained the gene for malic enzyme (sfcA) (Fig. 2). The LeuDH gene was then inserted into pHGW640.12 (Fig. 3), a pACYC184 derivative which also contained a rhamnose promoter and an sfcA gene, which latter was then deleted. The subcloning of the LeuDH gene from plasmid pAM5.22 (Fig. 2) into the target plasmid pAM10.1 (Fig. 4) was necessary in order to construct a two-plasmid system which requires two resistance markers for selection.

Table 4: Cloning results

Gene/vector	Cloned into plasmid	Restriction with	New designation	Fig.
FDH PCR fragment	pJOE4580.2	NdeI, AatII	pAM3.25	1
LeuDH PCR fragment	pJOE4625.1	BfrI, PstI	pAM5.22	2
LeuDH from pAM5.22	pHWG640.12	BfrI, BamHI	pAM8.21	3
pAM8.21	Without sfcA gene	MunI, PstI	pAM10.1	4

Fermenting the whole-cell catalyst

After HPLC analysis had shown that the FDH/LeuDH combination (*E.coli* JM109/pAM3.25/pAM10.1) achieved better results in converting trimethylpyruvate into tert-leucine than a comparative model system (malic enzyme/LeuDH on pAM5.22) in miniature-scale (1 ml) experiments in a thermoshaker, plasmids pAM3.25 and pAM10.1 were transformed into *E.coli* BW3110 since this strain is more suitable for fermentations. The intention was to use high cell density fermentation to prepare a sufficiently large biomass for all the following investigations using the model system. The fermentation was carried out without any antibiotic, with the preliminary cultures having been grown in the presence of antibiotic, at 30°C in a 30 l fermenter containing a final volume of 8 l. For this, the cells were initially grown at 30°C as a batch culture up to an OD600 = 50 and until the glucose had been completely consumed (approx. 22h). Gene expression was then induced by adding rhamnose, which had been sterilized by filtration, to a final concentration of 0.2%, while fed batch culture was started by automatically adding nutrient solution and minerals (feed I and feed II). Samples, whose OD and enzyme activities were

determined, using the respective activity tests in the latter case, were taken every two hours from the induction onward. The course of the OD, and of the activities, until fermentation was terminated are plotted against the time in Figure 5.

The fermentation was terminated 22h after the rhamnose induction since, despite increasing cell density, the activity of the FDH had stagnated and the cause of this was presumably plasmid loss or a reaction medium which was too acidic. The latter became apparent in the whole-cell reactions, in which the pH fell markedly (ΔpHmax = 0.8), as compared with a previously pH-regulated solution, when the moist biomass was added. The activities of the two enzymes reached 0.565 U/mg of total protein in the case of the LeuDH and 0.123 U/mg of total protein in the case of the FDH. The volume activities, based on the fermentation medium, were 32.77 U/ml for the LeuDH and 7.14 U/ml for the FDH. After the medium had been removed in a separator, the cell yield was 1.4 kg of moist biomass. The cells were stored temporarily at -20°C until being used as whole-cell catalyst.

Fermentation media

Preliminary culture:	2 × 200 ml
Preliminary culture medium:	cNa2SO4 × 10H2O = 2 g/l
	c(NH4)2SO4 = 2.675 g/l
	cNH4Cl = 0.5 g/l
	cK2HPO4 = 14.625 g/l
	cNaH2PO4 × 2H2O = 3.6 g/l

autoclave in 90% by vol. H2O

cglucose = 10 g/l, final concentration

(stock solution in H2O)

autoclave separately

1M MgSO4 solution, 2 ml/l
TES, 3 ml/l
Thiamine stock solution (10 g/l in H2O), 1 ml/l

Batch culture: Add inoculum (380 ml in which Cx = 12 g/l) containing glucose, MgSO4, TES and thiamine in an inoculation flask to the autoclaved batch medium

Batch medium (quantity taken for 8 l):

Na2SO4 × 10H2O	16 g
(NH4)2SO4	21.4 g
NH4Cl	4 g
K2HPO4	117 g
NaH2PO4 × 2H2O	28.8 g
(NH4) 2H-citrate	8 g

dissolve in 7.5 l of H2O and sterilize in a 30 l fermenter

Glucose monohydrate	220 g

dissolve in 500 ml of H2O and autoclave (25 g/l)

1M MgSO4 solution	16 ml
TES	24 ml
Thiamine solution (10 g/l)	8 ml

(sterilize the thiamine by filtration, autoclave the remainder)
pH 7.2, using H3PO4 and NH3

Fed batch feed:

I.	Glucose monohydrate	2750 g in 3.5 l of H2O
	autoclave	
	$MgSO_4 \times 7H2O$	98.5 g in 0.15 l of H2O
	autoclave	
	TES solution	0.5 l
	autoclave	
	Thiamine	2.5 g in 0.5 l of H2O
	sterilize by filtration	
	then combine in a feed flask	
II.	(NH4)2HPO4	396 g in 1 l of H2O, pH 7
	autoclave	

Feeds I and II are added using two separate pumps

pH: 7.2 (titrated with H3PO4 and NH3)

pO_2: approx. 50 kPa (regulated by the rotational speed of the agitator)

Trace element solution (TES):	CaCl2 × 2H2O	0.5 g
	ZnSO4 × 7H2O	0.18 g
	MnSO4×H2O	0.1 g
	Di-Na-EDTA	20.1 g
	FeCl3 × 6H2O	16.7 g
	CuSO4 × 5H2O	0.16 g
	CoCl2 × 6H2O	0.18 g
	H2O to 1 l	

Preparing L-tert-leucine using a whole-cell catalyst at 900 mM without metering (comparative example = synthesis example 1)

50 ml of an 0.9 M solution of trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia), which also contains 1 mM magnesium chloride and 1% (v/v) toluene, are added to 5.85 g of the biocatalyst (*E.coli* JM105 (pAM 3.25_10.1) biomass) and 7.95 g of ammonium formate (2.8 mol equivalents). The pH is adjusted to pH 7.0 at the beginning of the reaction and not regulated any further after that, resulting in the pH rising during the reaction. The reaction temperature is 30°C. After a reaction time of 8 h, a conversion of 24.6% is measured, with it not being possible to increase this conversion any further even after an additional 15 h of stirring.

Preparing L-tert-leucine using a whole-cell catalyst at approx. 0.9 M and employing fed batch metering (synthesis example 2)

23.84 g of ammonium formate (corresponding to 2.8 equivalents based on the total substrate quantity employed) and 17.55 g of the biocatalyst (E.coli JM105 (pAM 3.25_10.1) biomass) are initially weighed into a 250 l three-neck flask, after which 28.50 ml of deionized water and 150 µl of a 1M solution of magnesium chloride (corresponding to a 1 mM concentration based on the final volume) are added. When the reaction temperature of 30°C has been reached, the reaction is started by adding 7.50 ml of a 1.8 M solution of trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia). The pH is then adjusted to 7.0 by adding 32% ammonia. After that, in each case 7.50 ml of a 1.8 M solution of trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia) are firstly metered in twice after which different volumes of a 0.9 M solution of

trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia) are metered in five times, with all the additions taking place at defined time intervals. The time intervals, and the quantities which are in each case metered in, are given in the following metering table. The final volume is 150 ml and the total concentration of added substrate is 0.86 M, corresponding to a volumetric quantity of trimethylpyruvic acid of 112.5 g/l. A complete conversion (> 98% in accordance with HPLC) is observed after a reaction time of 24 h.

Metering table	Substrate solution	Substrate solution
Time (h)	ml (1.8 M)	ml (0.9 M)
0	7.5	0
0.5	7.5	0
1	7.5	0
2.5	0	15
4	0	17.5
5.5	0	20
6.5	0	22.5
7	0	24
Total volume of metered-in substrate solution	22.5	99

Preparing L-tert-leucine using a whole-cell catalyst at 1 M and employing continuous metering (synthesis example 3)

26.48 g of ammonium formate (corresponding to 2.8 equivalents based on the total quantity of substrate employed), 150 µl of a 1 M solution of magnesium chloride (corresponding to a 1 mM concentration based on the final volume) and 19.49 g of the biocatalyst (E.coli JM105 (pAM3.25_10.1) biomass) are initially weighed into a 250 ml three-neck flask, after which

30 ml of deionized water are added. The pH is then adjusted to 7.0 by adding 32% ammonia. After the reaction temperature of 30°C has been reached, a total of 120 ml of a 1.25 M solution of trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia) are added continuously at a flow rate of 0.2 ml/min over a period of 10 hours. The final volume is 150 ml and the total concentration of substrate employed is 1.0 M, corresponding to a volumetric quantity of trimethylpyruvic acid of 130.1 g/l. A conversion of 96% (in accordance with HPLC) is observed after a reaction time of 27 h.

Preparing L-tert-leucine using a whole-cell catalyst at 700 mM and employing fed batch metering (synthesis example 4)

2.55 g of sodium formate (corresponds to 2.5 mol/l based on final volume) are initially added to a conically shaped 100 ml reaction flask belonging to a STAT Titrino 718, after which 15 µl of a 1 M solution of $MgCl_2$ (corresponds to a final concentration of 1 mM) and 4.5 ml of a 1 M solution of TMP (pH 7, adjusted with 25% ammonia), and also 1.5% by vol. of toluene (based on the final volume), are added. The volume is made up to 15 ml with deionized H_2O. The reaction temperature of 30°C is kept stable, and controlled, by a closed-loop water circuit. 1 g of the biocatalyst moist biomass is resuspended in the substrate mixture and the pH is adjusted to 6.9 to 7 with 25% ammonia.

After pH 7.5 has been reached, 4.5 ml of the 1 M TMP solution (pH 7) are added repeatedly. In this connection, the pH falls by approx. $\Delta pH = 0.3$. As soon as pH 7.5 is reached, 4.5 ml of 1 M TMP solution are added once again. The addition of said volume of TMP is repeated 10× until the pH does not fall any further when TMP is added. In addition, 4 ml of a 4 M solution

of sodium formate (corresponds, without taking any reaction into consideration, to a concentration of 973 mM in the medium) are added in connection with the eighth addition of TMP. The final volume is 64 ml, with a volumetric final concentration (without taking the reaction into consideration) of trimethylpyruvic acid of 774 mM (100.6 g/l). Sodium formate is present in solution at a final concentration of 836 mM. HPLC showed that 92% of the trimethylpyruvic acid had been converted after only 6h.

The concentrations of the two substrates at the different addition points are listed in Table 5 below.

Time [t in min]	Concentration of trimethyl-pyruvic acid [mM]	Concentration of sodium formate [mM]	Second addition of sodium formate
0	300	2500	
45	461.54	1923.08	
60	562.5	1562.5	
75	631.58	1315.79	
90	681.82	1136.36	
105	720	1000	
120	750	892.86	
135	774.19	806.45	
150	736.36	972.73	x
180	756.30	899.16	
210	773.44	835.94	

Preparing a whole-cell catalyst which comprises a Bacillus cereus leucine dehydrogenase and a Bacillus subtilis glucose dehydrogenase

Strain preparation

Chemically competent E. coli DSM14459 (described in patent WO03/042412) cells were transformed with plasmid

pAM10.1 (Fig. 4, Seq. ID No. 10) (Sambrook et al. 1989, Molecular cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press). This plasmid carries a resistance to chloramphenicol (cat) and encodes a Bacillus cereus leucine dehydrogenase (ldh) (Stoyan, Tanja; Recktenwald, Achim; Kula, Maria-Regina. Cloning, sequencing and overexpression of the leucinee dehydrogenase gene from Bacillus cereus. Journal of Biotechnology (1997), 54(1), 77-80). The pAM10.1-transformed cells were then made chemically competent (Sambrook et al., 1989, Molecular cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press) and transformed with plasmid pNO4 (Fig. 6, Seq. ID No. 13). pNO4 carries a resistance to ampicillin (bla) and encodes a Bacillus subtilis glucose dehydrogenase (BS-GLUCOSE DEHYDROGENASE) (Glucose dehydrogenase from Bacillus subtilis expressed in Escherichia coli. I: Purification, characterization and comparison with glucose dehydrogenase from Bacillus megaterium. Hilt W; Pfleiderer G; Fortnagel P, Biochimica and biophysica acta (1991 Jan 29), 1076(2), 298-304). The genes for the leucine dehydrogenase and the glucose dehydrogenase are under the control of a rhamnose promoter (rhaP) (Stumpp, Tina; Wilms, Burkhard; Altenbuchner, Josef., A new L-rhamnose-inducible expression system for Escherichia coli. BIOspektrum (2000), 6(1), 33-36).

Preparing active cells

A single colony of E.coli DSM14459 (pAM10.1, pNO4) was incubated, at 37°C for 18 hours and with shaking (250 rpm), in 2 ml of LB medium (10 g of yeast extract/l, 5 g of tryptone/l, 10 g of NaCl/l) in the added presence of antibiotics (50 µg of ampicillin/l and 20 µg of chloramphenicol/ml). This culture was diluted 1:100 in fresh LB medium containing rhamnose (2 g/l) as inducer, added antibiotics (50 µg of

ampicillin/l and 20 µg of chloramphenicol/ml) and 1 mM ZnCl2, and incubated at 30°C for 18 hours with shaking (250 rpm). The cells were centrifuged (10 000 g, 10 min, 4°C), after which the supernatant was discarded and the cell pellet was used in biotransformation experiments either directly or after having been stored at -20°C.

Preparing L-tert-leucine using a whole-cell catalyst at 1 M and employing continuous metering (synthesis example 5)

9.98 g of the biocatalyst (E.coli DSM 14459 (pAM 10.1, pNO4) biomass) are initially taken up in 30 ml of water in a 250 l three-neck flask, after which 32.70 g of D glucose are added. The pH is then adjusted to 7.0 by adding sodium hydroxide solution (25% strength) and kept constant at this value during the reaction (total consumption: 13.11 ml). After the reaction temperature of 30°C has been reached, a total of 120 ml of a 1.25 M solution of trimethylpyruvic acid (pH 7.0, adjusted with 32% ammonia) are added continuously at a flow rate of 0.2 ml/min over a period of 10 hours. The final volume is approx. 165 ml and the total concentration of substrate employed is approx. 0.9 M, corresponding to a volumetric quantity of trimethylpyruvic acid of approx. 118 g/l. A conversion of > 97% (according to HPLC), and an enantioselectivity of > 99% ee for the product formed, are observed after a reaction time of 24h.